Vigna angularis inhibits mast cell-mediated allergic inflammation

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Abstract. The aim of the present study was to elucidate whether extracts of *Vigna angularis* (EVA) inhibit allergic inflammatory reactions and to elucidate the possible mechanisms of action. For the assessment of allergic inflammatory response, histamine release and the expression of pro-inflammatory cytokines from human mast cells (HMC-1) were examined. To identify the underlying mechanisms of action, intracellular calcium and the activation of nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs) were assayed. To confirm the effects of EVA *in vivo*, systemic and local allergic reaction mouse models were employed. EVA dose-dependently reduced phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-induced histamine release from mast cells. The inhibitory effects of EVA on the release of histamine from mast cells were mediated by the reduction of intracellular calcium levels. EVA decreased the PMACI-stimulated gene expression and secretion of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. The inhibitory effects of EVA on pro-inflammatory cytokines were NF-κB- and MAPK-dependent. In addition, EVA inhibited compound 48/80-induced systemic anaphylaxis and immunoglobulin E (IgE)-mediated cutaneous anaphylaxis. Our findings provide evidence that EVA inhibits mast cell-derived allergic inflammation, and suggest the possible therapeutic application of EVA in allergic inflammatory disorders.

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Introduction

Mast cells are broadly distributed throughout mammalian tissue and play various functions as regulators of allergic inflammation, such as asthma, atopic dermatitis and sinusitis (1). Immediate-type hypersensitivity (anaphylaxis) is mediated by the release of histamine in response to the antigen crosslinking of immunoglobulin E (IgE) bound to mast cells. Stimulated mast cells rapidly secrete pre-formed and *de novo* synthesized allergic mediators, such as histamine, cytokines and arachidonic derivatives (2). One of the allergic mediators, histamine, plays a major role in normal physiology and in pathophysiology, and regulates a variety of vital functions in the allergic inflammatory response (3,4).

The activation of mast cells leads to the phosphorylation of tyrosine kinase and the mobilization of internal calcium (5). These are followed by the activation of protein kinase C, mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB, as well as an increase in the expression of inflammatory cytokines (1). Activated mast cells release histamine and other inflammatory mediators, such as eicosanoids, proteoglycans and several pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6 and IL-13 (2,6). Although these inflammatory cytokines have beneficial effects on the host defense process, they cause pathological conditions when overexpressed. Therefore, the inhibition of the release of these inflammatory cytokines from mast cells is crucial to reducing allergic inflammatory symptoms.

Vigna angularis (azuki bean) is one of the largest crops in Asia and has long been used in alternative and complementary medicine in Korea, China and Japan. It has been prescribed for infection, edema and inflammation of the kidneys and bladder (7). *Vigna angularis* has been reported to exert tumor-suppressive, anti-diabetic, antioxidant and anti-inflammatory effects (7-9). In addition, Azuki bean seed coats, which are rich in polyphenols, have recently been reported to attenuate vascular oxidative stress in spontaneously hypertensive rats (10). However, the anti-allergic and anti-inflammatory effects of *Vigna angularis* have not yet been fully elucidated.

In the present study, we investigated the effects of extracts of *Vigna angularis* (EVA) on mast cell-mediated allergic inflammation using *in vitro* and *in vivo* models. The release of histamine and intracellular calcium levels were examined to clarify the mechanisms by which EVA inhibits the release of histamine from mast cells. The effects of EVA on pro-inflammatory cytokines and the role of NF-κB and MAPKs in these effects were investigated using human mast cells (HMC-1). In addition, to confirm the anti-allergic and anti-inflammatory effects of EVA in an *in vivo* system, systemic and local anaphylaxis mouse models were employed.

Materials and methods

Preparation of EVA. Vigna angularis was purchased from a herbal medicine store in Jeongeup, Korea. The authenticity of the plants was confirmed by Professor Y.H. Kim, at the College of Pharmacy of Chungnam National University, Daejeon, Korea. The *Vigna angularis* material (10 kg) was dried, pulverized to a fine powder and extracted twice with 95% EtOH at 70˚C for 4 h. The EtOH extract (120 l) of *Vigna angularis* was then passed through a 0.45-μm filter and vaporized in a rotary evaporator, yielding 100 g of residue. For the treatment of the cells, the stock solution (100 mg/ml) of EVA was dissolved in 100% DMSO and further diluted with Iscove's medium before use.

Reagents and cell culture. Compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187 (PMACI) were purchased from Sigma (St. Louis, MO, USA). The human mast cell line (HMC-1) was grown in Iscove's medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) at 37˚C in 5% CO_2. Passage 4-8 HMC-1 cells were used throughout the study.

Determination of histamine levels. The histamine levels in the HMC-1 cells and serum were measured using the *o*-phthaldialdehyde spectrofluorometric procedure as previously described (11). The HMC-1 cells (1x10^6 cells/ml) were pre-incubated with EVA for 30 min, and then incubated for 30 min with PMA (20 nM) and calcium ionophore A23187 (1 μM). The cells were separated from the released histamine by centrifugation at 400 x g for 5 min at 4˚C. The blood from the mice was centrifuged at 400 x g for 10 min and the serum was withdrawn to measure the histamine content.

Determination of intracellular calcium levels. The intracellular calcium levels were measured with the use of the fluorescence indicator, Fluo-3/AM (Molecular Probes, Eugene, OR, USA). The HMC-1 cells were pre-incubated with Fluo-3/AM for 30 min at 37˚C. After washing the dye from the cell surface, the cells were treated with EVA for 10 min prior to the addition of PMACI. The fluorochrome was excited at 488 nm, and the emission was filtered with 515 nm using a flow cytometer (BD Biosciences Pharmingen, San Diego, CA, USA).

RNA extraction and mRNA detection. Total cellular RNA was isolated from the cells (1x10^6/well in a 24-well plate) following stimulation with PMA (20 nM) and A23187 (1 μM) with or without EVA for 4 h using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The first-strand complementary DNA (cDNA) was synthesized using Superscript II reverse-transcriptase (Invitrogen, Carlsbad, CA, USA). A reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the mRNA expression of TNF-α, IL-6 and β-actin (internal control). The conditions for the reverse transcription and PCR steps were similar to those described previously (12). The amplified products were separated by electrophoresis on 2% agarose gels containing ethidium bromide, documented using a Kodak DC 290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT, USA). The band intensity was normalized to that of β-actin in the same sample.

Enzyme-linked immunosorbent assay (ELISA). The secretion of TNF-α and IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) according to a previously described method (13) with certain modifications. The HMC-1 cells were cultured in medium and resuspended in Tyrode buffer A. The cells were sensitized with PMACI for 8 h in the absence or presence of EVA. ELISA was performed by coating 96-well plates with 6.25 ng/well of monoclonal antibody with specificity for TNF-α or IL-6.

Western blot analysis. The HMC-1 cells were washed 3 times with PBS and resuspended in lysis buffer. The samples were electrophoresed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (14) and then transferred onto a nitrocellulose membrane. The p38 MAPK, ERK and JNK activation was determined using anti-phospho-p38, -ERK and -JNK antibodies (Cell Signaling Technology, Inc., Beverly, MA, USA). The nuclear and cytosolic p65 NF-κB and IκBα were assayed using anti-NF-κB (p65) and anti-IκBα antibodies, respectively (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunodetection was carried out using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc, Waltham, MA, USA).

Animals. The original stock of male imprinting control region (ICR) mice (6 weeks of age) was purchased from Dae Han Bio Link. Co., Ltd. (Chungbuk, Korea). The animals were housed 5 per cage in a laminar airflow room maintained under a temperature of 22±2˚C and a relative humidity of 55±5˚C throughout the study. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved the Animal Care and Use Committee at Kyungpook National University.

Systemic anaphylaxis. The mice were administered an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator, compound 48/80. EVA was dissolved in saline and orally administered at various doses (10, 50 and 250 mg/kg BW) 2 h prior to the injection of compound 48/80 (n=10/group). Mortality was monitored for 1 h after the induction of anaphylactic shock. After the mortality test, blood was obtained from the heart of each mouse to measure the serum histamine content.

Passive cutaneous anaphylaxis (PCA). An IgE-dependent cutaneous reaction was carried out as previously described (12). The PCA reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mouse tail vein. The anti-DNP IgE antibody and DNP-HSA were diluted in PBS. The mice were injected intradermally with 0.5 μg of anti-DNP IgE. EVA was orally administered at doses of 10, 50 and 250 mg/kg BW 2 h prior to the injection of anti-DNP IgE. After 48 h, each mouse (n=10/group) received an injection of 1 μg of DNP-HSA containing 4% Evans blue (1:4) via the tail vein. Thirty minutes after the challenge, the mice were sacrificed and the dorsal skin (diameter, 1 cm) was removed for measurement of the pigmented area. The amount of dye was then determined colorimetrically following extraction with 1 ml of 1 M potassium hydroxide (KOH) and 9 ml of a mixture of acetone and phosphoric acid (5:13). The absorbance intensity of the extraction was measured at 620 nm on a spectrophotometer (Shimadzu UV-1201; Shimadzu Corp., Kyoto Japan).

Statistical analysis. Statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC, USA). The effects of treatment were analyzed using analysis of variance, followed by Duncan's multiple range tests. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Effect of EVA on the release of histamine and intracellular calcium levels. First, we evaluated the effects of EVA on the release of histamine from PMACI-stimulated HMC-1 cells. The HMC-1 cells released high levels of histamine following stimulation with PMACI (15). Pre-treatment with EVA for 30 min reduced the release of histamine in a dose-dependent manner (Fig. 1A). To eludicate the mechanisms responsible for the reduction of histamine following treatment with EVA, we measured the levels of intracellular calcium. Calcium movements across the membranes of mast cells are critical to the release of histamine (16). When the HMC-1 cells were stimulated with PMACI, the intracellular calcium levels significantly increased. The pre-incubation of HMC-1 cells with EVA (100 μg/ml) decreased the intracellular calcium levels (Fig. 1B). BAPTA-AM (Molecular Probes) was used as a positive control. The concentration and duration of EVA treatment used in these experiments had no significant effect on the viability of HMC-1 cells (data not shown).

Effects of EVA on the expression and secretion of pro-inflammatory cytokines. We evaluated the effects of EVA on the gene expression and secretion of pro-inflammatory cytokines, such as TNF-α and IL-6 in the PMACI-stimulated HMC-1 cells. After the HMC-1 cells were pre-incubated with EVA for 30 min, they were then stimulated with PMACI for 4 h. As shown in Fig. 2A and B, EVA dose-dependently inhibited the PMACI-induced gene expression of TNF-α and IL-6. To confirm the effects of EVA on the gene expression of pro-inflammatory cytokines, culture supernatants were collected and the levels of TNF-α and IL-6 were measured by ELISA. EVA inhibited the secretion of TNF-α and IL-6 in the PMACI-stimulated HMC-1 cells (Fig. 2C and D).
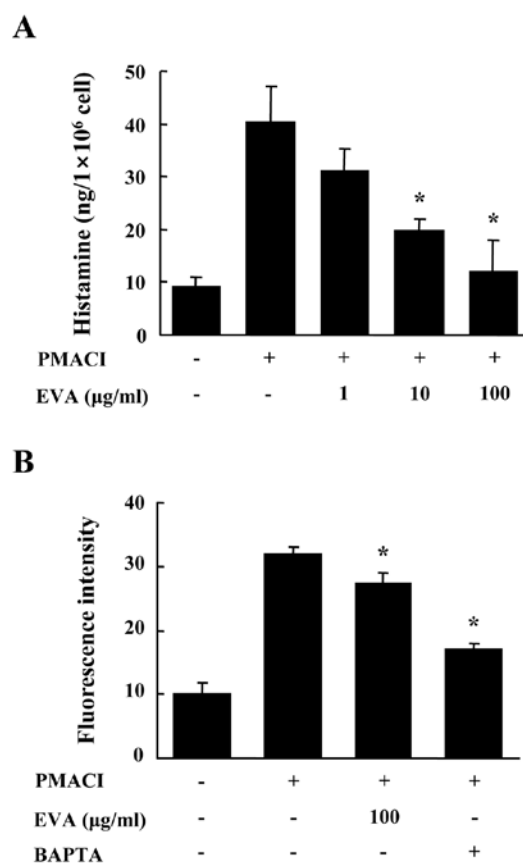

Figure 1. Effects of extracts of *Vigna angulari* (EVA) on the release of histamine and intracellular calcium levels in mast cells. Human mast cells (HMC-1) (1x10^6 cells/ml) were pre-incubated with EVA for 30 min prior to incubation with phorbol 12-myristate 13-acetate (PMA) (20 nM) and calcium ionophore A23187 (PMACI) (1 μM) for 30 min (A) or 10 min (B). Histamine content was measured using a spectrofluorometer. The level of relative fluorescence intensity was recorded using a flow cytometer. Each bar represents the mean ± SEM of 3 independent experiments. *P<0.05, significant difference of PMACI value.

Effect of EVA on the activation of NF-κB and MAPKs. To elucidate the mechanisms responsible for the reduction of cytokine levels, we examined the effects of EVA on the activation of the transcription factors, NF-κB and MAPKs. NF-κB is an important transcriptional regulator of inflammatory cytokines and plays a crucial role in immune and inflammatory responses (17). Stimulation of the HMC-1 cells with PMACI induced the degradation of IκBα and the nuclear translocation of p65 NF-κB after 2 h of incubation. EVA inhibited the PMACI-induced degradation of IκBα and the nuclear translocation of p65 NF-κB (Fig. 3). MAPK pathways also play a crucial role in the regulation of pro-inflammatory molecules (17). Previously, we demonstrated that PMACI activates all 3 types of MAPKs, such as p38, JNK and ERK within 15-30 min in HMC-1 cells (13). EVA markedly attenuated the PMACI-induced activation of all 3 types of MAPKs (Fig. 3). These data indicate that EVA decreases the expression of inflammatory cytokines by blocking the activation of NF-κB and MAPKs.

Effect of EVA on systemic and local anaphylaxis. To determine the effects of EVA on allergic reaction, an *in vivo* mouse model of systemic anaphylaxis was used. Compound 48/80 (8 mg/kg, BW) was used as a model of induction for a systemic
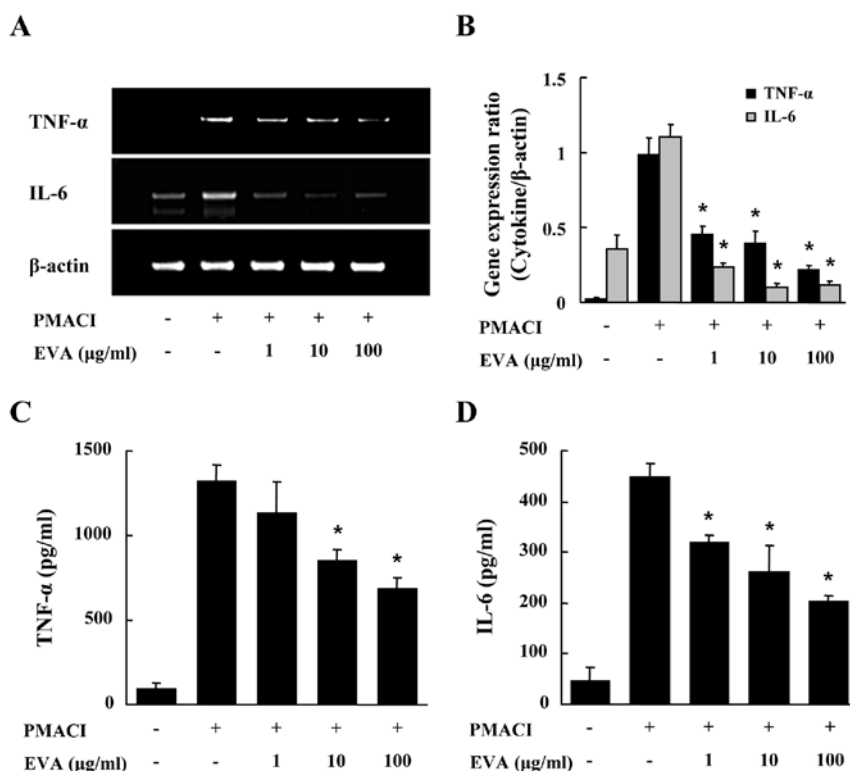
Figure 2. Effects of extracts of *Vigna angulari (*EVA) on gene expression and secretion of pro-inflammatory cytokines in mast cells. Human mast cells (HMC-1) (1x10^6 cells/ml) were pre-incubated with EVA for 30 min prior to incubation with phorbol 12-myristate 13-acetate (PMA) (20 nM) and calcium iono-phore A23187 (PMACI) (1 μM) for 4 h for RT-PCR and 8 h for ELISA. (A and B) Extraction and analysis of mRNA was performed as described in Materials and methods. The gene expression of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were determined by RT-PCR. Gel images were acquired using a Kodak DC290 digital camera, and densitometric analysis was performed using UN-SCAN-IT software. Each bar represents the mean ± SEM of 3 independent experiments. (C and D) The level of TNF-α and IL-6 in the supernatant was measured using ELISA and is presented as the mean ± SEM of 3 independent experiments. *P<0.05, significant difference of PMACI value.

Table I. Dose-dependent effect of EVA on compound 48/80-induced systemic anaphylaxis.

EVA treatment (mg/kg BW)	Compound 48/80 (8 mg/kg BW)	Mortality (%)
None (saline)	+	100
10	+	80
50	+	30
250	+	0
250	-	0

Groups of mice (n=10/group) were orally administered 200 μl of saline or EVA at various doses 2 h prior to the intraperitoneal injection of compound 48/80. Mortality (%) within 1 h following compound 8/80 injection is represented as the number of dead mice x100/total number of experimental mice. EVA, extracts of *Vigna angulari*; BW, body weight.

fatal allergic reaction. After the intraperitoneal injection of compound 48/80, the mice were monitored for 1 h, after which the mortality rate was determined. The injection of compound 48/80 into the mice induced fatal shock in 100% of the animals. When the animals were pre-treated with EVA (oral administration) at doses 10, 50 and 250 mg/kg (BW) for 2 h, the mortality rate was dose-dependently reduced (Table I). The effect of EVA on the compound 48/80-induced release of histamine in serum was also investigated. The injection of compound 48/80 induced a marked increase in the release of histamine in serum which was significantly inhibited by treat-ment with EVA at doses of 50 and 250 mg/kg BW (Fig. 4). Another way to test the anaphylactic reaction is to induce PCA. A local extravasation was induced by a local injection of IgE followed by antigenic challenge. EVA was orally administered at 10, 50 and 250 mg/kg (BW) 2 h prior to challenge with the antigen. EVA dose-dependently inhibited PCA (Fig. 5).

Discussion

Anaphylaxis is a life-threatening syndrome induced by the sudden systemic release of inflammatory mediators, such as histamine and various cytokines from mast cells (18). In this study, using *in vitro* and *in vivo* models, we demonstrate that EVA reduces mast cell-derived allergic inflammatory responses.

Histamine was originally considered as a mediator of acute inflammatory and immediate hypersensitivity responses. Previously, it was reported that histamine affects chronic inflammation and regulates several essential events of immune response, such as immune cell maturation, polarization and
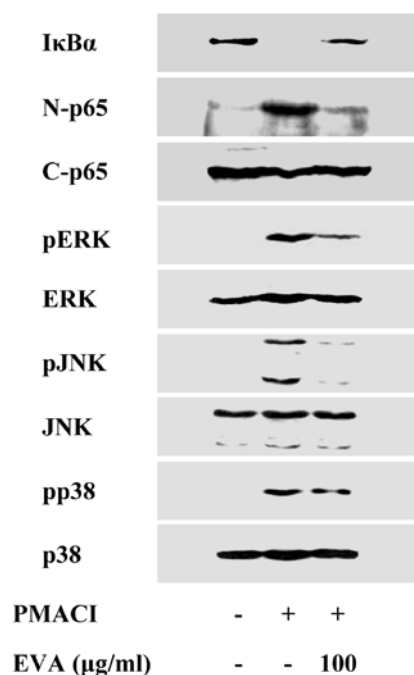
Figure 3. Effects of extracts of *Vigna angulari* (EVA) on the activation of nuclear factor (NF-κB) and phosphorylation of mitogen-activated protein kinases (MAPKs) in mast cells. Human mast cells (HMC-1) were pre-treated with EVA for 30 min prior to phorbol 12-myristate 13-acetate (PMA) (20 nM) and calcium ionophore A23187 (PMACI) (1 μM) stimulation. The nuclear translocation of NF-κB, IκBα degradation and the phosphorylation of MAPKs were assayed by western blot analysis (N-p65, nuclear p65 NF-κB; C-p65, cytoplasmic p65 NF-κB).

Figure 4. Effects of extracts of *Vigna angulari* (EVA) on the release of histamine in serum in systemic anaphylaxis. Groups of mice (n=10/group) were orally administered with 200 μl of saline or drugs at various doses 2 h prior to the intraperitoneal injection of compound 48/80 (8 mg/kg, BW). Blood was obtained from the heart of each mouse and histamine content was measured by spectrofluorometry. Each bar represents the mean ± SEM of 3 independent experiments. $^*P<0.05$, significant difference.

Figure 5. Effects of extracts of *Vigna angulari* (EVA) on passive cutaneous anaphylaxis (PCA). EVA was orally administered 2 h prior to challenge with the antigen. Each amount of dye was extracted as described in Materials and methods and measured by spectrophotometry. Each bar represents the mean ± SEM of 3 independent experiments. $^*P<0.05$, significant difference. DNP, anti-dinitrophenyl; HSA, human serum albumin.

lymphocyte responsiveness (19). Studies have established that the stimulation of mast cells with compound 48/80 or IgE initiates the activation of signal transduction pathways, which lead to the release of histamine. It has been demonstrated that compound 48/80 and other polybasic compounds are able to directly activate G proteins (20). Compound 48/80 increases the permeability of the lipid bilayer membrane by inducing a perturbation in the membrane. These data indicate that the increase in membrane permeability may be an essential trigger for the release of the mediator from mast cells. In this sense, anti-allergic agents having a membrane-stabilizing action may be desirable (21). EVA may stabilize the lipid bilayer membrane, thus preventing the compound 48/80-induced membrane perturbation.

Intracellular calcium is critical to the degranulation of mast cells. Calcium movements across the membranes of mast cells represent a major target for effective anti-allergic drugs, as these are essential events linking stimulation to secretion (22,23). The mode of action of EVA is possibly associated with the prevention of the release of histamine from mast cells due to the reduction in intracellular calcium levels. Our results showing an attenuation of intracellular calcium levels in mast cells following treatment with EVA are consistent with those from other reports. According to these observations, we strongly speculate that decreased intracellular calcium levels may be involved in the inhibitory effects of EVA on the release of histamine.
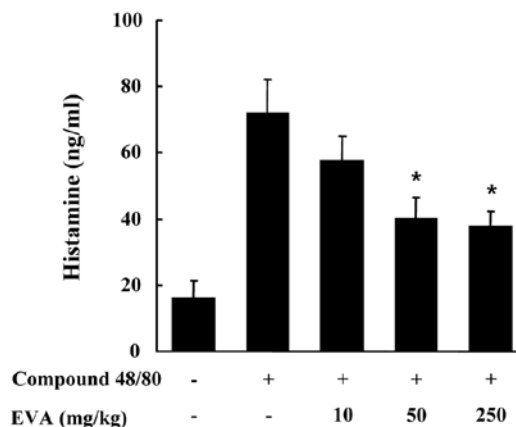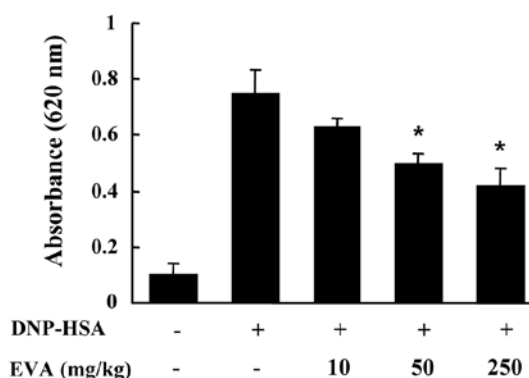
The HMC-1 cell line is useful for studying cytokine activation pathways (24). The various types of cytokines produced by HMC-1 cells with PMACI stimulation supports the well-recognized role of mast cells in immediate-type hypersensitivity. TNF-α and IL-6 play a major role in triggering and sustaining the allergic inflammatory response in mast cells. Mast cells are one of the major sources of TNF-α in the human dermis (25). TNF-α promotes inflammation, granuloma formation and tissue fibrosis and is considered to be an initiator of cytokine-related inflammatory states by stimulating cytokine production (26). TNF-α is involved in the survival of eosinophils, thereby contributing to chronic inflammation (27). IL-6 is also produced from mast cells and its local accumulation is associated with PCA (28). These reports indicate that the decrease in the levels of TNF-α and IL-6 in mast cells is one of the key indicators of reduced allergic inflammatory symptoms.

In our study, EVA decreased the elevated gene expression of TNF-α and IL-6 in mast cells. These data suggest that EVA exerts anti-inflammatory effects by inhibiting the production of inflammatory cytokines.

To evaluate the mechanisms behind the inhibitory effects of EVA on TNF-α and IL-6, we examined the effects of EVA on NF-κB. NF-κB regulates the expression of multiple inflammatory and immune genes and plays a critical role in chronic inflammatory diseases. The role of NF-κB activation and regulation of cytokine production in allergic inflammatory processes have been characterized (29). The activation of NF-κB requires the phosphorylation and proteolytic degradation of the inhibitory protein, IκBα, an endogenous inhibitor that binds to NF-κB in the cytoplasm (30). In our study, EVA decreased the degradation of IκBα and the nuclear translocation of p65 NF-κB. These results indicate that the inhibitory effects of EVA on inflammatory cytokines are due to the regulation of the NF-κB pathway.

The MAPK cascade is one of the important signaling pathways in immune responses (31). The MAPK signaling cascade regulates important cellular processes, including gene expression, cell proliferation, cell survival and death, as well as cell mobility (32). The expression of TNF-α and IL-6 is regulated by MAPKs (24). The precise signaling pathways among the 3 types of MAPKs, i.e., ERK, JNK and p38 remain unclear. However, the induction of inflammatory cytokine production requires the phosphorylation of all 3 types of MAPKs. In this study, the PMACI-induced phosphorylation of all 3 types of MAPKs was reduced by EVA. These data suggest that EVA exerts an inhibitory effect on all 3 types of MAPKs and downstream cytokine expression.

To confirm the effects of EVA in animal models, we evaluated the inhibitory effects of EVA on compound 48/80-induced systemic anaphylaxis and histamine release. These results indicate that mast cell-mediated immediate-type allergic reactions are inhibited by EVA. In addition, EVA administered to mice protects them against IgE-mediated PCA; the mouse model of PCA is one of the most important *in vivo* models of anaphylaxis in local allergic reactions. These finding suggest that EVA may be useful in the treatment of allergic diseases, particularly skin reactions. As we used whole extracts of *Vigna angularis* and not a purified single compound, the biological effects of the individual active components are not clear at this time. Nevertheless, EVA showed remarkable anti-allergic and anti-inflammatory effects. Therefore, we partitioned the *Vigna angularis* extracts based on anti-allergic and anti-inflammatory effects. Through the fractionation, we obtained 2 single compounds, oleanolic acid and oleanolic acid acetate. The effort to confirm the actual role of these compounds on mast cell-mediated allergic inflammation is ongoing in our laboratory.

In conclusion, the present study demonstrates that EVA significantly reduces mast cell-mediated allergic inflammation in *in vitro* and *in vivo* models. We suggest that EVA reduces the release of histamine through the modulation of intracellular calcium. EVA inhibits the expression of inflammatory cytokines by suppressing the activation of NF-κB and MAPKs. We provide evidence that EVA may be used in the prevention or treatment of mast cell-mediated allergic inflammatory diseases.

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